

Mycotoxin production from fungi isolated from grapes

L. Abrunhosa¹, R.R.M. Paterson², Z. Kozakiewicz², N. Lima¹ and A. Venâncio¹

¹Centro de Engenharia Biológica-IBQF, Universidade do Minho, Braga, Portugal, and ²CABI Bioscience UK Centre, Egham, UK

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Aims: In order to assess the potential for producing mycotoxins, fungi were isolated from wine producing grapes.

Methods and Results: The isolates were identified and *Penicillium expansum*, the most well recognized mycotoxin producer, was analysed for mycotoxin production by TLC. Many of the strains produced patulin and/or citrinin, often depending on whether they were grown on a grape or yeast extract sucrose media.

Conclusions: Citrinin was produced by all strains grown in the yeast extract sucrose medium, but only one strain (from 51) was able to produce this compound in grape juice medium.

Patulin was produced in the yeast extract medium by 20 strains and in grape juice medium by 33 strains.

Significance and Impact of the Study: The presence of mycotoxins in wine producing grapes is discussed. Grapes contamination with patulin seems not to contribute to wine contamination, and no ochratoxin producing fungi was identified.

INTRODUCTION

There is increasing concern about the presence of mycotoxins in commodities. These compounds are produced by particular species of fungi. Patulin (Scott *et al.* 1977) and ochratoxin A (Majerus and Otteneder 1996; Zimmerli and Dick 1996) are mycotoxins that have been reported in grapes and grape products such as wine. However, ochratoxin A has not recently been found in Portuguese wine (Festas *et al.* 2000). Patulin is produced most notably by the fruit-rotting fungus, *P. expansum*, whereas ochratoxin A is associated with *Penicillium verrucosum* and *Aspergillus ochraceus*. Patulin causes gastrointestinal problems, skin rashes, and is known to be mutagenic. Ochratoxin A is carcinogenic and the European Union is considering imposing regulatory limits in wine. Patulin has been demonstrated to be present in apple and grape juices (Scott *et al.* 1977; Moss 1998), although it is absent from ciders as it is degraded by the fermentation process. There is a recommended limit for patulin in apple juice in the UK of 50 µg l⁻¹. Citrinin is also produced by *Penicillium expansum*, but was not found in apple and grape juices (Scott *et al.* 1977). The production of grapes and products such as wine is important to the Portuguese

economy and to countries which import these commodities. Port wine is a particularly important export commodity and is produced from grapes from the Douro region of Portugal. This paper reports on work to isolate fungi from grapes from two wine-producing regions of Portugal, and to analyse recognized mycotoxin-producing fungi to assess the potential for mycotoxin contamination of wine.

MATERIALS AND METHODS

Grape sampling and preparation

Grapes were harvested from three vineyards from Ponte do Lima in the Vinhos Verdes region, and a vineyard from Vila Nova de Foz Côa in the Douro region. Sampling took place in mid-August in the Vinhos Verdes region when the first signs of fungal invasion can be observed. Samples were also taken in late September or the first days of October, corresponding to the harvest time of the 1999 season in the Vinhos Verdes and Douro regions. A vineyard of high altitude was selected for the Douro region samples; the conditions are not optimal for Port wine but the greatest mould growth is observed here. Grapes with and without visual signs of fungal invasion were harvested.

Fungi were isolated from the skins and interiors of the grapes (the pulp). Each grape surface was sterilized for 2 min in sodium hypochloride, containing 0.4% available

Correspondence to: Prof. A. Venâncio, Centro de Engenharia Biológica-IBQF, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal (e-mail: avenan@deb.uminho.pt).

chloride, and washed twice with sterile distilled water. The skin sterilization steps were checked for efficacy by pressing onto Dichloran Rose Bengal Chloramphenicol (DRBC) (Oxoid) and on Dichloran Rose Bengal Yeast Extract Sucrose (DRYES) both supplemented with chloramphenicol (0.01% (w/v)) (Frisvad 1983).

The pulp was separated from the skin under aseptic conditions and then macerated in 1 ml sterile solution of 0.1% (w/v) peptone. This was vigorously shaken in a vortex for 1 min. Macerated pulp (0.2 ml) was plated onto DRBC and DRYES. The macerated pulp was diluted (10×) with 0.1% peptone solution and plated out, as above, if numbers of fungi were too high to count.

For skin analysis, intact grapes were cut under aseptic conditions and treated as described above, but without the sterilization step. In this set of experiments, dilutions of 10^{-2} and 10^{-3} were plated on DRBC and DRYES as described for macerated pulp.

Grapes from each vineyard that had visual signs of fungal growth (rotten) were also analysed. These grapes were cut and macerated with 0.1% peptone solution as above. Dilutions of 10^{-4} and 10^{-5} were plated onto DRBC and DRYES. All plates were incubated at 25°C in the dark.

Isolation and identification of fungal species

Fungal colonies were transferred to individual slants of Malt Extract Agar 1 (Oxoid) as they became visible at 4 × magnification. This medium was also used to maintain the isolates at 4°C. Taxonomic identification of all isolates was achieved through macroscopic and microscopic observation in accordance with guidelines published for each genus. The identification of the penicillia strains was made according to

Pitt (1979). Five of the identified species were confirmed and deposited at CABI Bioscience (Egham, UK). Most of the strains have been deposited in the Micoteca da Universidade do Minho (Braga, Portugal) culture collection.

Metabolite production by *P. expansum*

All *P. expansum* strains were grown on two media: yeast extract sucrose (YES) medium and a grape juice (GJ) medium (1 kg grapes were crushed in 1 litre distilled water and boiled for 2 h. The grape extract was filtered and sterilized by autoclaving, then 15 g agar were dissolved in 800 ml distilled water and autoclaved. GJ medium was prepared by mixing 200 ml grape extract with the 800 ml agar solution). The cultures were analysed by removing agar plugs, as described in Paterson and Bridge (1994). Thin layer chromatography was undertaken in a saturated chamber, using silica gel 60 TLC plates without indicator (Merck) and a TEF solvent system (toluene, ethyl acetate and formic acid, 5:4:1). The spray reagent was either MBTH (3-methyl-2-benzothiazoline hydrazone hydrochloride, 5 g l⁻¹) with heating for 15 min at 110°C, or *p*-anisaldehyde (0.5% v/v) with heating for 8 min at 105°C. Griseofulvin, patulin and ochratoxin A (all Sigma) were used as standards.

RESULTS AND DISCUSSION

Eight genera were isolated from the grapes (Table 1). *Penicillium*, *Cladosporium* and *Botrytis* prevailed in the Douro region and *Cladosporium* and *Botrytis* were dominant in the Vinhos Verdes region. One *Aspergillus* strain was identified from white grapes from the Douro region, although it was not identified to the species level and

Table 1 Fungi isolated from the most representative white and red castes from Vinhos Verdes and Douro regions (percentage indicated in brackets)

Fungi	Vinhos Verdes		Douro	
	White castes	Red castes	White castes	Red castes
<i>Alternaria</i> spp.	5 (12%)	6 (18%)	4 (3%)	2 (3%)
<i>Aureobasidium pullulans</i>	2 (5%)	0	4 (3%)	5 (7%)
<i>Aspergillus</i> spp.	0	0	1 (1%)	0
<i>Botrytis</i> spp.	11 (27%)	9 (26%)	45 (35%)	23 (30%)
<i>Cladosporium</i> spp.	12 (29%)	12 (35%)	32 (25%)	23 (30%)
<i>Fusarium</i> spp.	6 (15%)	6 (18%)	3 (2%)	1 (1%)
<i>Penicillium</i> spp.	5 (12%)	1 (3%)	40 (30%)	22 (29%)
<i>P. aurantiogriseum</i>	0	0	0	1
<i>P. brevicompactum</i>	1	0	8	1
<i>P. expansum</i>	0	0	31	20
<i>P. glabrum</i>	0	1	0	0
<i>P. mimioluteum</i>	0	0	1	0
<i>P. spinulosum</i>	3	0	0	0
<i>P. thomii</i>	1	0	0	0
<i>Tricothecium roseum</i>	0	0	1 (1%)	0
Total	41 (100%)	34 (100%)	130 (100%)	76 (100%)

was not tested for mycotoxin production. However, it was not considered to be *A. flavus* or *A. ochraceus*, which produce aflatoxins and ochratoxin A, respectively. *Penicillium expansum* was identified only from the Douro region and was isolated in large amounts from white and red grapes. The grapes were collected from vineyards that may be used to produce Port. It is interesting to note that *P. expansum* was not isolated from the Vinho Verde grapes. This may be due to the fact that the weather conditions during this season were poor, and heavy *Botrytis* infection was observed. It is known that the cultural conditions for the two varieties are significantly different, and as the Vinho Verde grapes are also more acidic, these conditions may not be conducive to the otherwise ubiquitous *P. expansum*.

Each strain was different in its capacity to produce patulin and/or citrinin, depending on whether it was grown on GJ or YES (Table 2). A small percentage (20%) of *P. expansum* isolates were positive for patulin on YES agar, whereas they were all positive for citrinin. Citrinin is recognizable as a green streak with a Rf of 34. When growing on GJ medium, a higher percentage (65%) of isolates were positive for patulin and only one strain was positive for citrinin. The difference is probably due to the chemical composition of the media. This situation is similar to that in apple juice and grape products in which citrinin is not formed or is unstable (Scott *et al.* 1977), although patulin is common. It would be interesting to test the non-producing strains for potential patulin production with the gene probe for the patulin metabolic pathway reported in Paterson *et al.* (2000). A metabolite reported in Abo-Dahab *et al.* (1996) from *P. expansum* was similar to ochratoxin A. However, analysis undertaken here indicated that ochratoxin A has a higher Rf value than this metabolite, hence confirming that it was not ochratoxin A. The isolation of *P. brevicompactum* is interesting as this fungus was isolated frequently in a study on apples in the UK, although *P. expansum* was more common (Paterson *et al.* 2000). When DNA from the *P. brevicompactum* isolates was gene-probed for the patulin metabolic pathway, a PCR product(s) was detected. However, patulin (and citrinin) was not detected, as was the case in the present study. As would be expected, ochratoxin A was not detected. A report of ochratoxin A from a *P. brevicompactum* strain in

Paterson and Kimmelmeier (1989) requires confirmation by other techniques using the same strain. The cultures were positive for brevianamide A in YES and GJ media.

In conclusion, ochratoxin A-producing fungi were not isolated from the grapes. However, it appears that patulin contamination of Douro grapes may be a possibility. The observation that *P. expansum* was not isolated from Vinho Verde grapes is interesting but requires further investigation; it may be due to different cultivation and environmental factors.

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Table 2 and citrinin production by the 51 strains *Penicillium expansum* grown in two different media

Number of strains	GJ medium		YES medium	
	Patulin	Citrinin	Patulin	Citrinin
10 (20%)	+	–	+	+
1 (2%)	+	+	–	+
22 (43%)	+	–	–	+
18 (35%)	–	–	–	+